EXHIBIT B

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The Role of Oxidative Stress in Salt-Induced Hypertension

Mohamed A. Bayorh, Agaba A. Ganafa,
Robin R. Socci, Natalia Silvestrov, and Imad K. Abukhalaf

Background: Impairment of endothelial function during hypertension is associated with increased production of superoxide radicals and reduced antioxidants. We investigated the involvement of oxidative stress in Dahl salt-sensitive (SS) and salt-resistant (SR) rats.

Methods: For a 2-week period, male rats were fed either high salt (HS; 8% sodium chloride) or low salt (LS; 0.3% sodium chloride) diets. Before and weekly on the diets, mean arterial pressure (MAP) and heart rate were measured by tail-cuff plethysmography. At the end of the experiment, plasma and tissue samples were collected for analysis of nitric oxide, prostacyclin, glutathione, and isoprostane.

Results: The MAP was increased in SS rats on HS diet, but not in those on a LS diet or in SR rats on either diet. Plasma levels of nitric oxide were reduced in SS rats on HS diet. Plasma prostacyclin levels in SS rats on either diet were lower than SR on LS diet. Increased dietary salt reduced plasma prostacyclin levels in SR, but not in SS rats. Plasma total 8-isoprostane was elevated in both SS and SR rats on HS diet compared with either strain on LS diet. Plasma levels of total glutathione were reduced in SS compared with SR rats, regardless of the level of dietary salt intake. The whole blood ratio of reduced-to-oxidized glutathione (GSH/GSSG) as well as the kidney total glutathione were lower in SS rats on HS diet. Aortic superoxide production in both strains on HS diet was increased compared with the animals on LS diet.

Conclusions: These data suggest that HS diet may indirectly induce endothelial dysfunction through intermediate mechanisms that are associated with oxidative stress. Am J Hypertens 2004;17:31–36 © 2004 American Journal of Hypertension, Ltd.

Key Words: Dahl rats, glutathione, oxidative stress, endothelium-derived relaxing factors.

Oxidative stress may play a critical role in the pathogenesis of hypertension, as well as other cardiovascular disorders such as atherosclerosis and myocardial infarction.[1] In the Dahl salt-sensitive (SS) hypertensive rat there is evidence of an elevated number of circulating leukocytes that produce superoxide compared with its normotensive control, the Dahl salt-resistant (SR) rat.[2] Also, oxidative stress induced by glutathione depletion in normal rats has been shown to cause and maintain severe hypertension.[3] Consistent with this notion, supplementation with antioxidant substances has been suggested to reduce blood pressure (BP) in hypertensive individuals and provide protection against oxidative cardiovascular injury.[4,5] Several studies have also reported that levels of some free radical scavengers such as vitamin E and superoxide dismutase are depressed in hypertensive patients.[6–8] However, a range of antioxidant defenses have evolved to detoxify reactive oxygen species, a major one of which is the glutathione redox cycle.[9] Glutathione is the most abundant nonprotein intracellular thiol, with multiple roles as an antioxidant agent.[1] Reduced glutathione (GSH) acts to scavenge reactive oxygen species as well as to regenerate other antioxidants from their oxidized forms.[10] In this process, glutathione is converted to its oxidized form (GSSG), which must be reduced by the combination of glutathione reductase and NADPH. Thus, an index of cellular oxidative events is the ratio of the levels of the reduced and oxidized forms of glutathione.

The pathogenesis of hypertension in the SS has also been associated with suppression of nitric oxide (NO) release and impairment of endothelial function.[11] Therefore, endothelial dysfunction is an important contributor to

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From the Departments of Pharmacology and Toxicology (MAB, AAG, RRS, IKA), Space Medicine and Life Sciences Research Center (MAB, IKA), and Clinical Research Center (IKA), Morehouse School of Medicine, Atlanta, Georgia.

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Address correspondence and reprint requests to Dr. Mohamed A. Bayorh, Department of Pharmacology/Toxicology, Morehouse School of Medicine, 720 Westview Drive, S.W., Atlanta, GA 30310-1495; e-mail: bayorh@msm.edu

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the pathogenesis of hypertension in the SS because vascular endothelial cells are known to generate endothelium-derived relaxing factors, such as NO and prostacyclin, which mediate vasodilation. Nitric oxide plays a critical role in the maintenance of arterial pressure responses and peripheral vascular resistance and contributes to the resting vascular tone of conductance and resistance arteries.[12] Prostacyclin, one of the primary cyclooxygenase products released from blood vessels, promotes systemic and renal vasodilation and excretion of salt and water.[13]

Reactive oxygen species, such as superoxide radicals, form vasoconstrictor isoprostanes from nonenzymatic peroxidation of arachidonic acid[14,15] and may inactivate NO by generating peroxynitrite.[3,16] Plasma total isoprostane levels are considered to be a sensitive and reliable marker of oxidative stress.[17]

This study investigates the cardiovascular effects of dietary salt and alterations in the plasma and tissue levels of total glutathione, plasma total isoprostane, as well as plasma NO and prostacyclin in Dahl SS and SR rats. Also as a measure of oxidative stress, the ratio of reduced-to-oxidized glutathione (GSH/GSSG) in whole blood and the aortic production of superoxide anion were determined.

Methods

Experimental Design

Male Dahl SS and SR rats (4 to 5 weeks) were obtained from Harlan Sprague-Dawley (Indianapolis, IN) and acclimatized for 1 week in the animal facility that has 12-h light/dark cycles with the temperature controlled at 21° to 23°C. Normal rat chow and water were made available ad libitum. After acclimatization, the animals were put in individual cages and fed either a low- (0.3% NaCl) or high-salt (8% NaCl) diet, in groups of six for each strain, for 2 weeks. Before (basal) and weekly thereafter, indirect mean arterial pressure and heart rate were measured. After these measurements, each animal was anesthetized (70 mg/kg ketamine and 10 mg/kg xylazine, intramuscularly) and the carotid artery and jugular vein cannulated with PE-50 tubing containing heparinized saline (20 U/mL). The cannulas were externalized in the posterior cervical region, occluded with a metal plug, and flushed with heparinized saline every 12 h.

Indirect BP Measurement in Conscious Rats

Tail-cuff plethysmography (Rat Tail Blood Pressure Monitor and Universal Oscillograph, Harvard Apparatus Inc., Holliston, MA) was used to measure mean arterial pressure. Heart rate was measured from the arterial pulse wave at the same time.

Collection and Storage of Blood Samples

Twenty-four hours after surgery, blood samples (2.0 mL) for NO and prostacyclin measurements were collected by free flow through the polyethylene cannula in the right carotid artery into chilled heparinized and indomethacin (100 mmol/L)-rinsed (for prostaglandin samples) tubes and replaced with an equal volume of saline. For glutathione and total isoprostane, blood samples (5 mL) were withdrawn by a cardiac puncture from all animals under anesthesia (ketamine/xylazine mixture) before being killed. For determination of reduced (GSH) and oxidized (GSSG) glutathione, 0.5 mL of ice-cold 0.2 mol/L boric acid/10% perchloric acid solution (BA/PCA) was mixed with 0.5 mL of whole blood and processed for plasma. All blood samples were centrifuged at 3000*g* for 25 min at 4°C. For isoprostane, butylated hydroxytoluene was added to 1.0 mL of plasma to give a final concentration of 0.005% (v/v). For total glutathione, 1.0 mL of ice-cold BA/PCA solution was added to 1.0 mL of plasma, vortexed, incubated for 5 min at room temperature, and then centrifuged at 3000*g* for 3 min. All plasma samples were frozen in aliquots and stored at −80°C until assayed.

Tissue Harvesting

Immediately after cardiac puncture, the heart and the kidneys were harvested from all the animals and frozen in liquid nitrogen and stored at −80°C.

Measurement of Plasma Levels of Prostacyclin, Total 8-Isoprostane, NO, and Glutathione

After purification according to the manufacturer's instructions, plasma levels (in picograms per milliliter) of prostacyclin (as 6-keto-$PGF_{1\alpha}$) and total 8-isoprostane (free plus esterified in lipoproteins) were measured using enzyme immunoassay kits (Cayman Chemical Corp., Ann Arbor, MI). Plasma NO (as nitrates + nitrites) levels (in micromoles per liter) were measured spectrophotometrically (540 nm) by a microplate assay using the Greiss reagent (Cayman Chemical Corp., Ann Arbor, MI). The protocols of Abukhalaf et al[18] and Kirlin et al[19] were used to analyze plasma total glutathione levels (in micromoles per liter) by HPLC after derivatization. Whole blood levels of reduced and oxidized glutathione were analyzed by the protocol of Jones et al[20] and expressed as a reduced-to-oxidized ratio.

Measurement of Heart and Kidney Total Glutathione

Heart and kidney tissues were mixed 1:1.5 (w/v) with cold phosphate-buffered saline and homogenized for about 3 min—at 1 min on and 1 min off intervals. The mixture was then centrifuged at 3500*g* for 30 min. The supernatant was collected and frozen at −80°C until assayed. Total glutathione levels were measured as described for plasma and expressed as in micromoles per liter per gram of tissue.

Measurement of Aortic Superoxide Production

Briefly, aortas were thawed, cleaned of connective tissue, and homogenized (glass/glass) in ice-cold HEPES buffer (containing 25 mmol/L HEPES, 1 mmol/L EDTA, and 0.1 mmol/L phenylmethylsuflonyl fluoride) (1:10, w/v). After centrifugation at 6000*g* for 5 min at 4°C, the supernatant was collected, frozen, and stored at −80°C. Protein was determined in an aliquot of each homogenate by the Bio-Rad method.[21]

Superoxide production was measured in the following reaction mixture (0.2 mL total): 10 μmol/L dihydroethidium, 0.5 mg/mL salmon testes DNA, 10 μg of homogenate protein, and the appropriate substrate for either NADH/NADPH oxidase (0.1 mmol/L NADH or NADPH), mitochondrial respiratory enzymes (5 mmol/L succinate), or xanthine oxidase (0.1 mmol/L xanthine). This mixture was incubated on a 96-well microplate for 30 min at 37°C. Ethidium/DNA fluorescence was measured at an excitation of 475 nm and an emission of 610 nm on a Cytofluor II fluorescence plate reader (Biosearch Products, Bedford, MA). The data was expressed as fluorescence units per minute per milligram of protein.

Chemicals

Chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). The 0.3% and 8% NaCl diets were obtained from Harlan Teklad (Madison, WI). Kits for determinations were obtained from Cayman Chemical Co. (Ann Arbor, MI).

Statistical Analysis

Values are reported as mean ± SE (SEM), where *n* refers to the number of rats used. Statistical significance ($P < .05$) was evaluated using either Student *t* test, or for multiple groups, analysis of variance (ANOVA) followed by the Tukey-Kramer multiple comparison test.

Results

BP and Heart Rate

Salt-sensitive rats, starting with a basal mean arterial pressure of 91 ± 1 mm Hg ($n = 12$), showed a significant pressure elevation over the 2-week period on high salt diet (121 ± 4 mm Hg, $n = 6$) compared with those of rats on a low salt diet (101 ± 2 mm Hg, $n = 6$; Fig. 1A). In contrast, SR rats showed no significant difference during the 2-week period from their basal mean arterial pressure (86 ± 2 mm Hg), regardless of dietary salt intake (Fig. 1A). There were no significant changes in heart rate for either rat strain regardless of the level of dietary salt intake (Fig. 1B).

Plasma and Tissue GSH

As depicted in Fig. 2, plasma total glutathione levels were significantly reduced in SS rats (LS, 0.3 ± 0.04 μmol/L; HS, 0.4 ± 0.08 μmol/L) compared with SR rats (LS, 36 ± 4 μmol/L; HS, 40 ± 3 μmol/L), regardless of the level of dietary salt intake. Kidney glutathione was significantly lower in SS rats on a high salt diet (1.7 ± 0.1 μmol/L) compared with SS rats on a low salt diet (2.1 ± 0.2 μmol/L) or SR rats on either diet (LS, 2.4 ± 0.2 μmol/L; HS, 2.3 ± 0.1 μmol/L). There was no significant difference in heart glutathione levels.

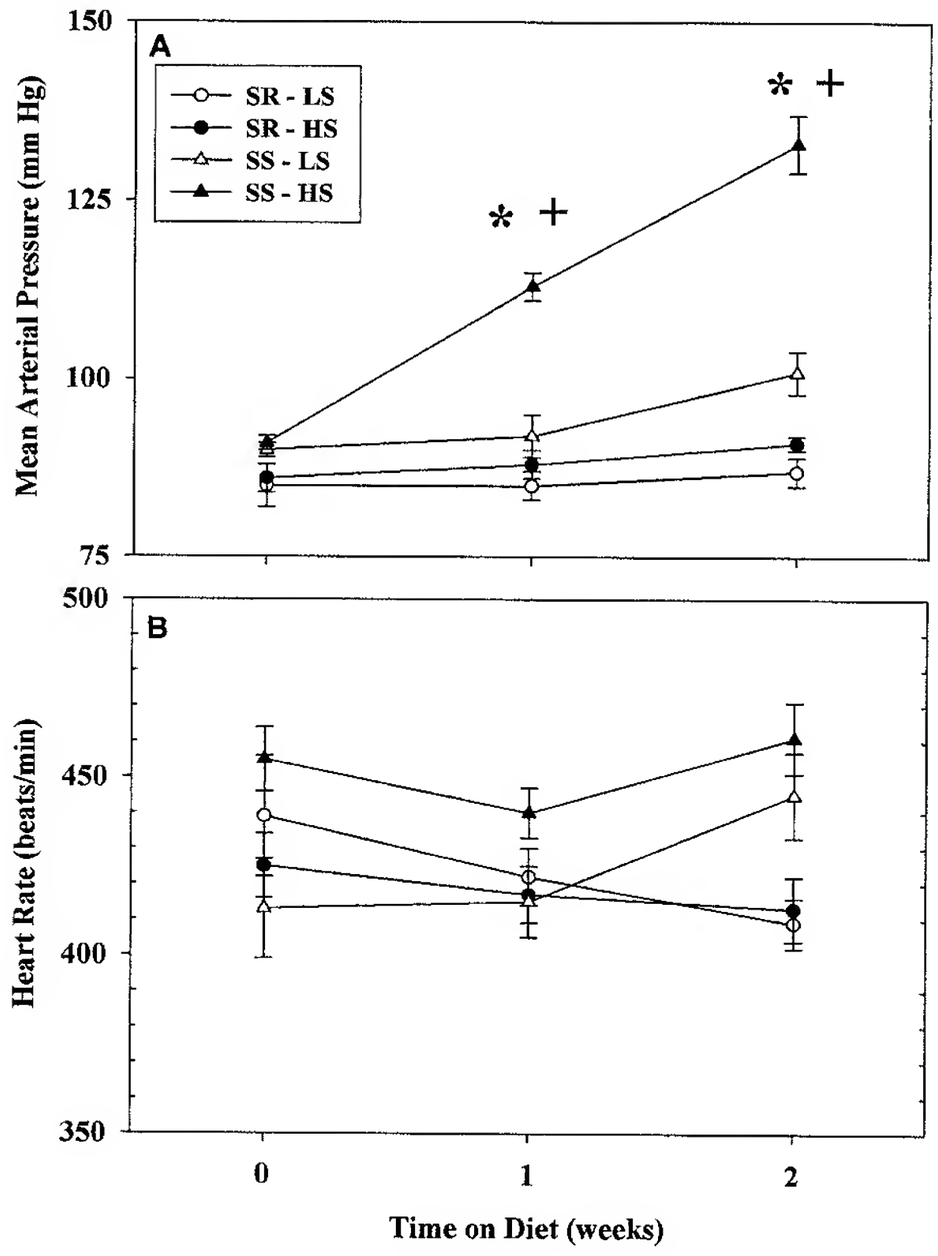

FIG. 1. Effect of dietary salt on mean arterial blood pressure (**A**) and heart rate (**B**) in Dahl rats. Data are represented as mean ± SEM for six animals per group. Significant effect ($P < .05$) due to dietary salt is denoted by the **asterisk**, whereas a significant difference due to the rat strain is denoted by a **plus** symbol. LS = low salt; HS = high salt.

Whole Blood GSH/GSSG Ratio

Whole blood GSH/GSSG ratio in SS rats on a high salt diet (4.9 ± 0.2) was reduced compared with all other groups (SS–LS, 7.1 ± 0.3; SR–LS, 6.3 ± 0.5; SR–HS, 7.3 ± 0.6) as shown in Fig. 3.

Plasma Levels of NO and Prostacyclin

Plasma NO levels in SR rats were unaffected by the level of dietary salt intake (LS, 26 ± 5 μmol/L; HS, 32 ± 5 μmol/L). There was no significant difference in NO levels between the strains when placed on a low salt diet (SS, 34 ± 4 μmol/L). In SS rats, increasing the level of dietary salt intake produced a reduction in plasma NO (21 ± 2 μmol/L) as shown in Fig. 4A. Plasma levels of the vasodilator prostacyclin in SS rats on either diet (LS, 255 ± 42

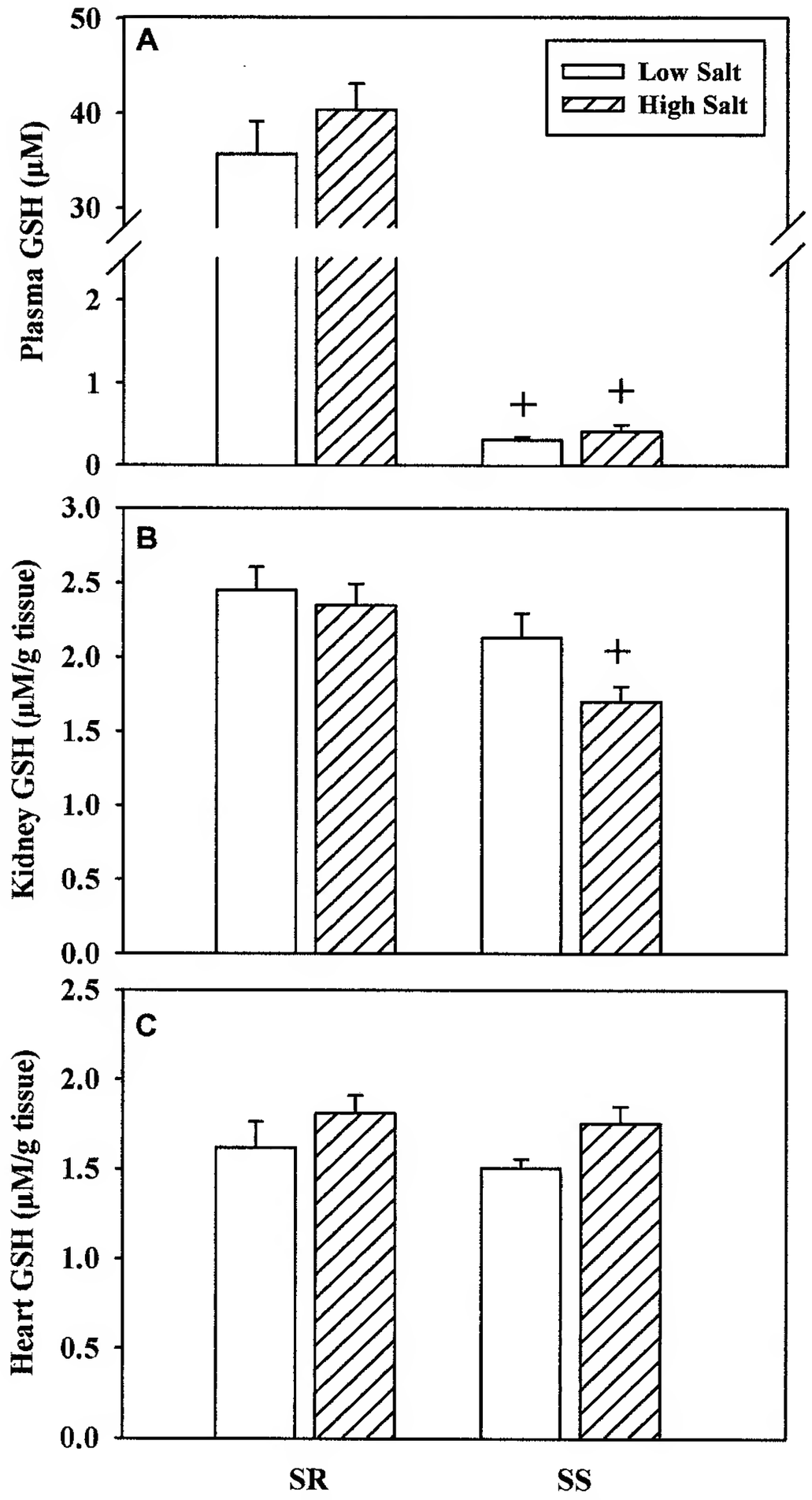

FIG. 2. Effect of dietary salt on plasma (**A**), kidney (**B**), and heart (**C**) levels of total glutathione (GSH) in Dahl rats. Data are represented as mean ± SEM for six animals per group. Significant effect ($P < .05$) due to rat strain is denoted by a **plus** symbol.

pg/ml; HS, 190 ± 33 pg/mL) were significantly lower than SR rats on a normal salt diet (402 ± 24 pg/mL). An increased level of dietary salt intake resulted in a reduction of plasma prostacyclin levels in SR rats (190 ± 33 pg/mL), but did not alter them in SS rats (Fig. 4B).

Aortic Superoxide Production

Superoxide production, by way of NADH and NADPH, in aortas from rats of either strain on HS diet was higher than that of aortas from rats on LS diet, as shown in Fig. 5.

Plasma Levels of Isoprostane

Plasma levels of total isoprostane were significantly elevated in both strains of rats on a high salt diet (SS, 271 ± 48 pg/mL; SR, 293 ± 78 pg/mL) compared with those on a low salt diet (SS, 99 ± 16 pg/mL; SR, 85 ± 10 pg/mL), as shown in Fig. 6.

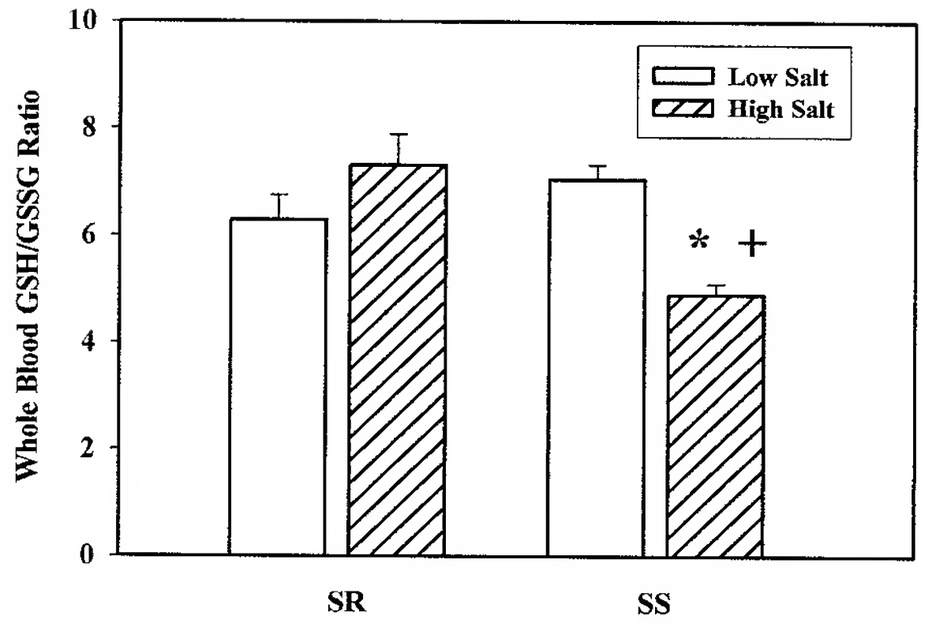

FIG. 3. Effect of dietary salt on the whole blood GSH:GSSG ratio in Dahl rats. Data are represented as mean ± SEM for six animals per group. Significant difference ($P < .05$) due to dietary salt is denoted by an **asterisk**, whereas a significant effect due to rat strain is denoted by a **plus** symbol.

Discussion

Data from the present study show that salt-induced hypertension is associated with oxidative stress, involving sig-

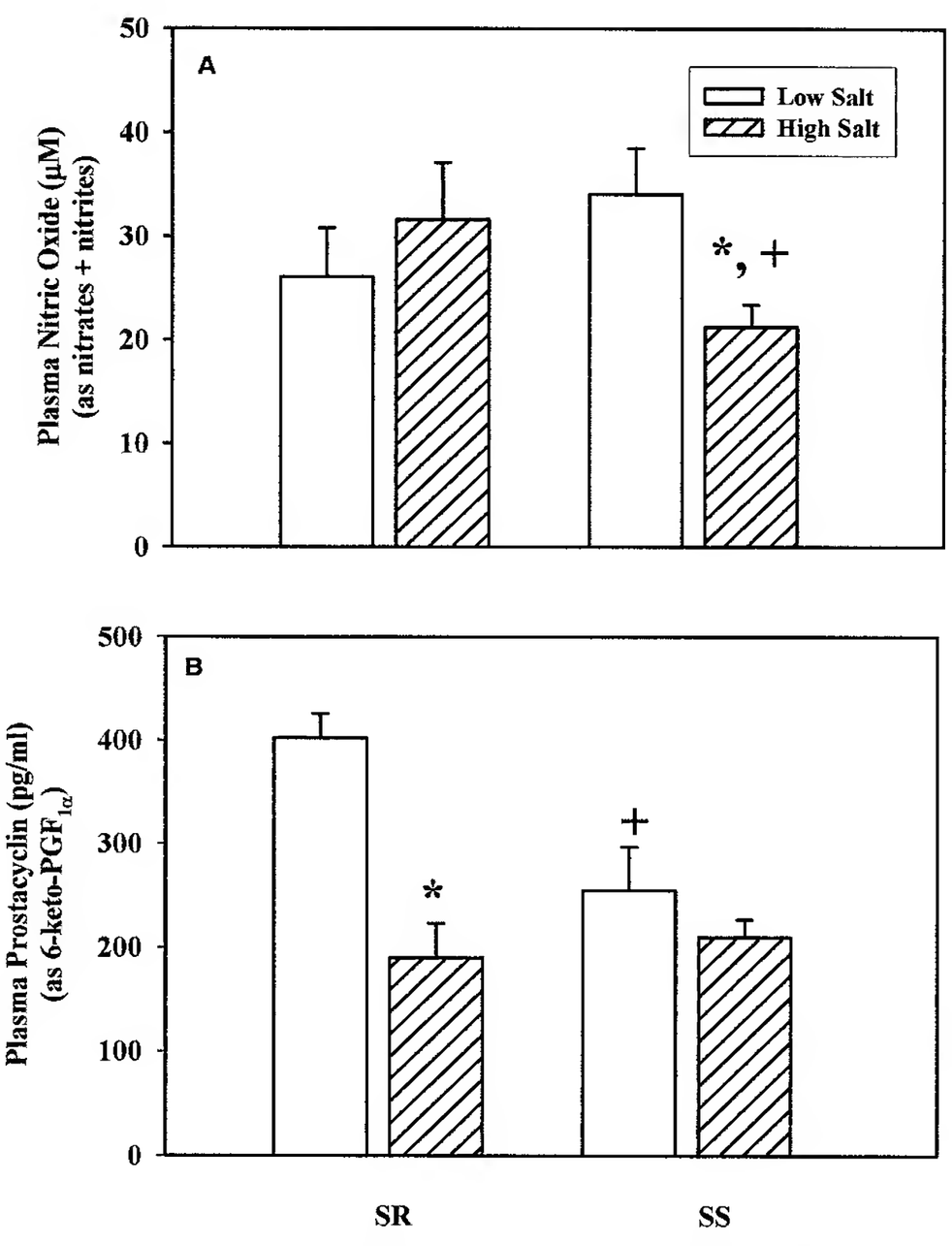

FIG. 4. Effect of dietary salt on plasma levels of NO (**A**) and prostacyclin (**B**) in Dahl rats. Data are presented as mean ± SEM for six animals per group. Significant difference ($P < .05$) due to dietary salt is denoted by an asterisk, whereas a significant effect due to rat strain is denoted by a **plus** symbol.

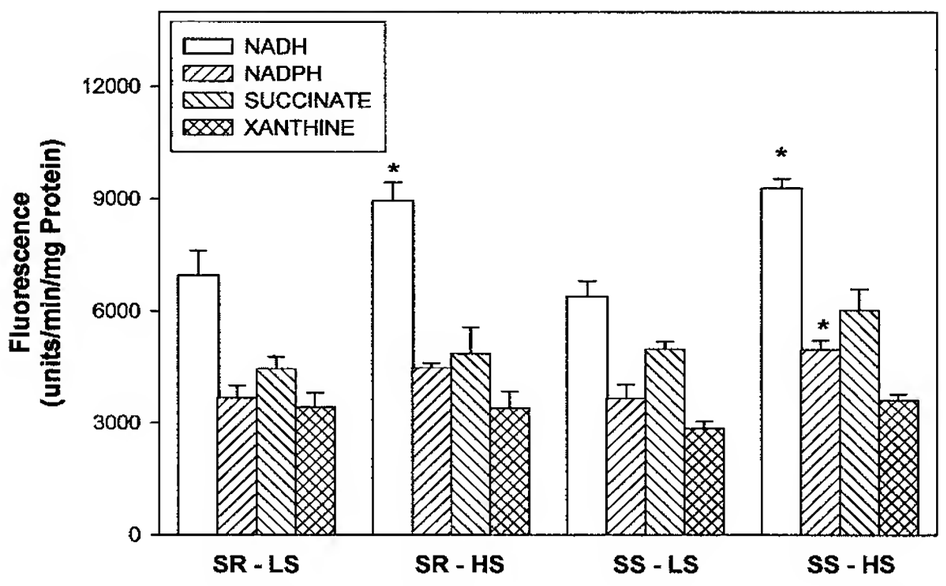

FIG. 5. Effect of dietary salt on superoxide production, by different pathways, in aortas from Dahl rats. Data are represented as mean ± SEM for five animals per group. Significant difference ($P < .05$) due to dietary salt is denoted by an **asterisk**. Abbreviations as in Fig. 1.

nificant changes in glutathione homeostasis and endothelial dysfunction. In SS rats fed a high salt diet, the increase in mean arterial pressure (BP) was associated with reductions in the plasma and kidney glutathione content and the whole blood GSH/GSSG ratio. Plasma total glutathione levels were markedly reduced in the SS rats regardless of the level of dietary salt intake compared to the SR rats. Likewise, in a recent study, it has been demonstrated that the animal model of the Dahl SS rat has a compromised antioxidant status, defined by significantly decreased glutathione peroxidase and glutathione activity compared with the SR rat.[22] Also, plasma levels of isoprostane, a recognized marker of oxidative stress, were elevated in both strains when placed on a high salt diet. Thus, the alterations in glutathione and isoprostane levels correlate significantly with the development of high BP in the SS rat but not the SR during the 2-week treatment period. This observation suggests that oxidative stress and the associated maladaptive changes may occur independent of excessive increase in BP because the SR rats without significant elevation in BP demonstrated a significant increase in isoprostane levels. Consistently, aortic

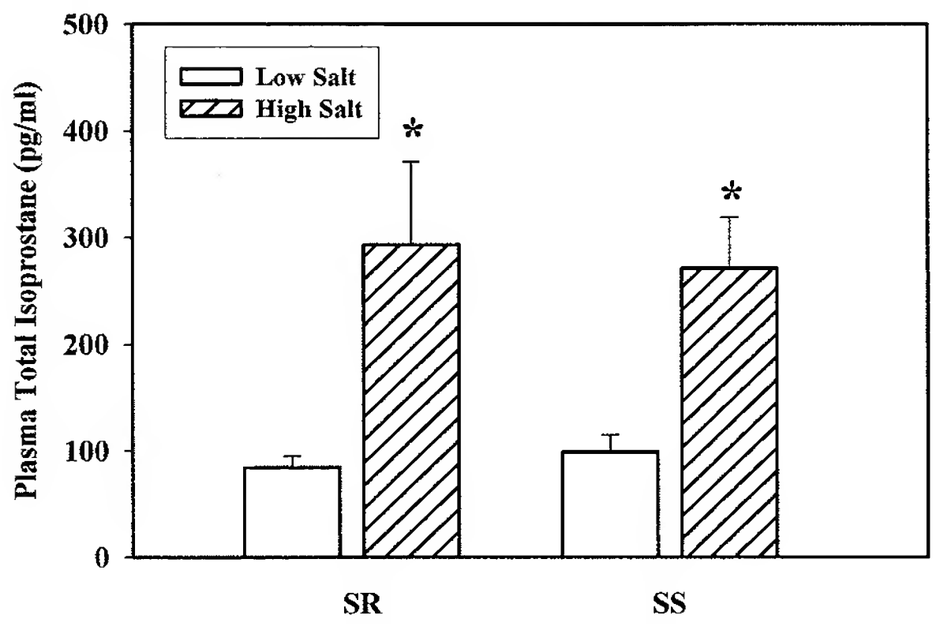

FIG. 6. Effect of dietary salt on plasma total isoprostane levels in Dahl rats. Data are represented as mean ± SEM for six animals per group. Significant difference ($P < .05$) due to dietary salt is denoted by an **asterisk**.

superoxide production was increased in both the SS and SR fed a high salt diet by way of NADH, and it increased in the SS through both NADH and NADPH. During normal metabolism, reactive oxygen species (ROS) are continuously produced in the vascular endothelium by NADPH oxidase, xanthine oxidase, and mitochondrial enzymes. Also, in renal tissues NADH and NADPH that are substrates for the enzyme NADPH oxidase have been demonstrated to be the major source of superoxide formation.[23] Under physiologic conditions, ROS are kept at low noncytotoxic levels by cellular and extracellular antioxidant defense systems. In vascular tissues, for example, the activity of superoxide dismutase lowers the levels of superoxide free radicals to the picomolar range.[24] During oxidative stress, however, ROS-mediated signaling may be directly involved in regulating vascular tone through physiologic processes, such as receptor activation, pressure, and flow.[25]

Endothelial dysfunction, assessed in terms of endothelium-derived factors, was manifested by reductions in the plasma levels of NO and prostacyclin. The results are consistent with our previous findings where we showed reductions in plasma NO in SS rats on high salt compared to those on a low salt diet.[26] Previous studies have shown that during oxidative stress, superoxide anion reacts with NO at a higher rate than it does with superoxide dismutase to form peroxynitrite, a potent cytotoxic oxidant; therefore, reducing the available NO.[27,28] In this study, the increase in superoxide anion and reduction in both plasma GSH and whole blood GSH/GSSG ratio in the SS rats, but not the SR, was associated with a reduction in plasma NO levels. Thus, oxygen free radicals may affect both vascular resistance, by inactivating NO and causing arteriolar vasoconstriction and elevation of peripheral resistance, and may serve as trigger mechanisms for lesion formation. A reduction of plasma prostacyclin levels was also observed in SR rats with increased levels of dietary salt intake. It should be noted that SS rats on either level of dietary salt intake had plasma prostacyclin levels similar to SR rats on high salt diet. It is possible that the alterations in glutathione levels and increases in superoxide anion associated with a high salt diet have an inhibitory effect on prostacyclin formation. In related studies, using buthionine-sulfoximine, the pressor response secondary to oxidative stress was associated with significantly reduced plasma prostacylin levels.[29,30] Alterations in the GSH/GSSG system are capable of activating or inactivating many enzymes that may affect prostanoid synthesis. For instance, changes in the enzyme activities of glutathione peroxidase and glutathione reductase after antioxidant therapy with vitamin E have been linked to an increase in the GSH/GSSG ratio and prostacyclin levels.[31]

Certain caveats, however, that may limit the interpretation of our results should be considered. First, we did not measure endothelial function directly. Thus the causal relationship between salt diet, oxidative stress, and endothelial function was expressed in terms of plasma levels of

endothelium-derived factors, NO, and prostacyclin. Second, endothelial damage may be a secondary effect related to other possible factors and intermediate mechanisms not studied here. In humans, salt sensitivity has been associated with decreased cystosolic-free magnesium levels, which is a possible determinant of glutathione production.[32] Several investigators have demonstrated that magnesium metabolism is altered by dietary salt, and magnesium deficiency increases the susceptibility of the cardiovascular system to oxidative stress.[33–35]

These limitations notwithstanding, we have found a significant correlation between oxidative stress and impaired endothelial function, in terms of vasodilator availability, in the development of salt-induced hypertension. Oxidative stress induced by high salt is greater in the SS than the SR rats. Thus, the finding of markedly low levels of plasma glutathione in the SS rat on either diet is extremely important and warrants further investigation.

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